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SIMPLIFIED METHODS FOR OBTAINING PURIFIED OOCYSTS FROM MICE AND FOR GROWING *CRYPTOSPORIDIUM PARVUM* IN VITRO

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ABSTRACT: Seven- to 8-day-old Arc/Swiss mice were infected with 100,000–120,000 *Cryptosporidium parvum* oocysts. At 8 days postinfection (PI) the jejunum, ileum, cecum, colon, and rectum were removed. Using a simple extraction procedure and purification by Ficoll gradient centrifugation, we rountinely obtained between 3–6 million and up to 15 million purified oocysts per mouse. For in vitro cultivation, purified oocysts were pretreated in a low pH (2.5–3) 0.5% trypsin solution for 20 min, resuspended in supplemented RPMI-1640 containing glucose 0.1 g (5.55 mM), sodium bicarbonate 0.3 g, bovine bile 0.02 g, folic acid 25 μg, 4-aminobenzoic acid 100 μg, calcium pantothenate 50 μg, ascorbic acid 875 μg, penicillin G 10,000 U and streptomycin 0.01 g per 100 ml, and 1% fetal bovine serum (pH 7.4 before filtration), and used to inoculate confluent monolayers of the human adenocarcinoma cell line HCT-8. Incubation was in a candle jar at 37 C. We tested numerous supplements to RPMI-1640, different pHs, and atmospheric conditions and found the parameters described above produced the greatest parasite numbers in vitro. We obtained significantly superior growth of *C. parvum* grown in HCT-8 cells using the conditions described above than in culture conditions described previously.

Cryptosporidium is a protozoan parasite with a worldwide distribution. *Cryptosporidium parvum* is the species found infecting a range of domestic and wild animals, and humans (Casemore, 1990; Fayer et al., 1990; O'Donoghue, 1995) between which cross-transmission can occur (reviewed by O'Donoghue, 1995). The parasite primarily invades the epithelium of the gastrointestinal tract of infected hosts causing asymptomatic infections or an acute or chronic cholera-like diarrheal disease, which can be life-threatening or cause failure to thrive in infants, children, and immunocompromised individuals (Isaacs et al., 1985; Current, 1989; Guarino et al., 1995). The serious nature of cryptosporidiosis in high risk groups is compounded by the lack of curative treatment strategies. In this respect, a major limiting factor has been the lack of a cultivation system for biochemical studies and for the systematic assessment of potential anticryptosporidial chemotherapeutic agents.

An important prerequisite for the establishment of a suitable cultivation system for *C. parvum* is a reliable and easily obtainable source of oocysts, a simple oocyst purification procedure, and identification of optimal conditions for growth. Although many studies have reported the in vitro development of *C. parvum* in various cell lines, most of these studies have found that, despite some development of *C. parvum,* the percentage of cells infected is low. The work of Upton, Tilley, and Brillhart (1994a, 1994b, 1995) and Upton, Tilley, Nesterenko, and Brillhart (1994) has identified a number of important growth additives, culture conditions, and a cell line that has been shown to greatly enhance growth of *C. parvum* in vitro. We have examined a number of these variables and have optimized conditions in our laboratory that produce superior growth of *C. parvum* in vitro. In addition, we report a simple method for obtaining large quantities of purified oocysts from mice suitable for in vitro cultivation and for molecular and biochemical studies.

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MATERIALS AND METHODS

Cryptosporidium isolates

The *C. parvum* isolate used for the majority of in vivo and in vitro manipulations in this study was originally obtained from a calf in Millicent, South Australia in 1993 and has subsequently been passaged through mice in our laboratory. This *C. parvum* isolate was designated C1. *Cryptosporidium parvum* isolates from humans were obtained from individuals in Western Australia and used to inoculate mice (Table I) and 1 isolate (H2) used for in vitro cultivation. The calf isolate and several human isolates have been previously characterized by Morgan et al. (1995).

Infection of mice and collection of oocysts

We have modified the method described by Current (1990) for recovering oocysts from mice. Seven- to 8-day-old ARC/Swiss mice (Animal Resources Centre, Murdoch, Western Australia) were inoculated with 100,000–120,000 oocysts per os using a plastic tube (diameter 0.7 mm) attached to a 1-ml syringe. Mice began shedding oocysts on day 4 postinfection (PI). Fecal samples were collected per rectum on days 6 and 7 PI and placed into dH_2O (distilled water) (≈0.25 ml/mouse) containing 0.02% Tween-20 (BDH, Poole, England) and stored at 4 C. On day 8 PI, mice were killed (CO_2 exposure) and the jejunum, ileum, cecum, colon, and rectum removed, placed in dH_2O/0.02% Tween-20 (approximately 4 ml/mouse), and dissected into smaller segments. Intestinal and fecal material was pooled and Sputasol (0.005 g/ml of suspension; Oxoid, Hampshire, England) added before the suspension was thoroughly homogenized at 4 C. The homogenate was left at RT (room temperature) for 90–120 min on a rotary mixer before centrifuging at 2,000 *g* for 10 min. The suspension was washed twice with cold dH_2O/0.02% Tween-20 by centrifuging at 2,000 *g* for 8 min. An ether extraction was performed by resuspending the oocyst-containing pellet first with dH_2O/0.02% Tween-20, adding ether (2 ml of ether per 8 ml of suspension) and mixing vigorously (20–30 sec) before centrifuging at 2,000 *g* for 8 min. The supernatant layers were removed, and the pellet was washed twice in cold dH_2O as described above. Additional oocysts were recovered by remixing the supernatant layers following the ether extraction and recentrifuging as described above to pellet and wash oocysts. Oocysts were resuspended in cold sterile PBS (phosphate-buffered saline) and stored at 4 C until further purification.

Purification of oocysts collected from mice

Oocysts were further purified using a modification of the method described by Lumb et al. (1988). Modifications included reduction of the number of Ficoll gradients from 6 to 2 and changing the centrifugation speed and time. Gradients were formed by layering 2.5 ml of a 0.5% and 1.0% Ficoll 400 (Pharmacia, Uppsala, Sweden) solution pre-

TABLE I. Results of mice inoculated with different human (and calf) isolates of *C. parvum*.

Isolate code	Sex and age of patient*	Age and strain of mouse	No. of mice infected	Oocyst dose/mouse	Feces†	Gut‡ (oocysts/mouse)
PAH	F, 2 yr	7-day ARC/Swiss	5	20,000	–	–
PAH	F, 2 yr	7-day BALB/c	3	20,000	–	–
PP	M, 3 yr	6-day BALB/c	5	20,000	–	–
KW	F, 1 yr	6-day BALB/c	7	50,000	–	–
084	NA	6-day BALB/c	5	100,000	–	–
GM	M, 43 yr	6-day BALB/c	5	10,000	–	–
H5	M, 7 yr	6-day BALB/c	5	100,000	+	+
CNA	M, 1 yr	8-day ARC/Swiss	8	80,000	+–2+	5,000
H11	NA	8-day ARC/Swiss	8	100,000	–	–
H10	F, 7 yr	8-day ARC/Swiss	8	100,000	–	–
H6	M, 3 yr	8-day ARC/Swiss	8	100,000	–	–
H2	NA, 6 yr	7-day ARC/Swiss	30	100,000	+–4+	2–6 million
H4	M, 8 yr	7-day ARC/Swiss	8	100,000	–	–
H1	M, 2 yr	7-day ARC/Swiss	4	100,000	–	–
H1	M, 2 yr	7-day ARC/Swiss	4	1,000,000	–	–
C1	Calf	7-day ARC/Swiss	>100	100,000	+–4+	3–6 million

* NA = information not available.
† Feces examined by preparing direct fecal smears and staining with safranin.
‡ Gut examined by preparing smears from cecal contents and staining with safranin. Oocysts quantitated after purification as described in the Materials and Methods.

pared in PBS containing 16% sodium diatrizoate (ICN, Aurora, Ohio) into a 10-ml centrifuge tube starting with the 1.0% Ficoll layer at the bottom. Two to 4 ml of oocyst suspension was layered onto the top of a cold Ficoll gradient (4 C) and tubes centrifuged (swinging bucket rotor) for 15 min at 1,000 *g* at RT. Oocysts were collected from the PBS/0.5% Ficoll interphase and washed twice with cold dH_2O as described above. Additional oocysts were also recovered following Ficoll gradient centrifugation by resuspending pellets in PBS and recentrifuging on fresh gradients and collecting oocysts as described above. Purified oocysts were resuspended in cold sterile PBS and 15 µl of an antibiotic solution (gentamycin 5.0 mg/ml, lincomycin 4 mg/ml, ampicillin 10 mg/ml) added per ml of resuspended oocysts before storage at 4 C.

In vitro cultivation

Excystation of oocysts and culture medium: Excystation was carried out by exposing 0.1–1-ml aliquots of purified oocysts to 9 ml of freshly prepared excystation medium consisting of filter-sterilized (0.22 µm filter) dH_2O (pH 2.5–3) containing 0.5% trypsin (1:250; Difco, Detroit, Michigan) and incubating at 37 C for 20 min, with mixing every 4–6 min. The excystation suspension was centrifuged at 1,500 *g* for 4 min at RT, oocysts resuspended in RPMI-1640 (Sigma, St. Louis, Missouri) basal medium (RPMI-1640bm) containing sodium bicarbonate 0.3 g, bovine bile 0.02 g (Sigma), penicillin G 10,000 U, and streptomycin 0.01 g per 100 ml (adjusted to pH 7.4 before filtration).

Preparation and infection of host cells: We evaluated the cell lines MDCK, Caco-2, human fibroblasts (HF, Health Laboratories of Western Australia), HT-29, a colon cancer cell line (LIM1215; Ludwig Institute for Cancer Research, Victoria, Australia; Whitehead et al., 1985), and HCT-8 (ATCC; CCL244). In preliminary testing, we found that the human ileocecal adenocarcinoma cell line HCT-8 supported the best growth of *C. parvum* and was subsequently used for routine cultivation.

HCT-8 cells were maintained in 25-cm² tissue culture flasks in RPMI-1640 supplemented with 1% fetal bovine serum (FBS; Gibco, Auckland, New Zealand), antibiotics (as above), and sodium bicarbonate (as above) at 37 C in a 5% CO_2 incubator. For subculture, HCT-8 cells were grown in RPMI-1640 containing 10% FBS. HCT-8 cultures that had recently reached confluent monolayers grown in 25-cm² flasks or 24 well plates were inoculated with *C. parvum* by removing existing medium and replacing it with RPMI-1640bm containing pretreated oocysts (see above) and incubating at 37 C in a candle jar for between 80 and 90 min. The medium was then removed and replaced with RPMI-1640 containing different supplements and incubated for 72–120 hr at 37 C under different atmospheres (see below). We routinely used an oocyst inoculum of between 2,000 and 5,000 oocysts/cm² of culture area.

Growth supplements and culture conditions: The following supplements were added to RPMI-1640 and evaluated for their effects on cryptosporidial growth: 0.01, 0.015, 0.02, 0.03, 0.04, 0.05, 0.06, and 0.08 g/100 ml bovine bile; 0.1, 0.25, 0.3, and 0.6 g/100 ml biosate (BBL, Cockeysville, Maryland); 0.3 g/100 ml lactalbumin (Sigma); 0.3 g/100 ml yeast extract (BBL); 0.3 g/100 ml trypticase (BBL); 0.3 g/100 ml tryptone (Oxoid); 0.3 g/100 ml tryptone (Difco); 0.3 g/100 ml peptone (Sigma); 0.3 g/100 ml *Trichomonas* medium (Oxoid); 0.02 g/100 ml sodium taurocholate (BDH); 0.1, 0.25, 0.3, 0.4, and 0.5 g/100 ml sodium bicarbonate; 0.24 and 0.1 U/ml insulin; 1 and 2 mM sodium pyruvate; 50 and 100 µM mercaptoethanol; 0.5 and 1 mM cysteine; 0.01 and 0.02 g/100 ml ascorbic acid (Sigma); nonessential amino acids (Flow, McLean, Virginia); 5.55, 6.25, 12.5, 25, and 50 mM glucose; 1% Ficoll 400; 1 mM glutathione; 15 and 20 mM Hepes; 1% hypoxanthine–thymine medium supplement (Sigma); 1, 2, 5, and 10% FBS; 2, 5, and 10% new born calf serum (CSL, Parkville, Australia), and omitting serum. The complete medium was as described by Upton et al. (1995), in addition to the vitamin mixture: 100 µg/100 ml folic acid (ICN), 400 µg/100 ml 4-aminobenzoic acid (ICN), 200 µg/100 ml calcium pantothenate (ICN), 3,500 µg/100 ml ascorbic acid (Sigma), added at 20, 25, 50, and 100% original concentrations. Many of these supplements were also tested in combination and under different atmospheric conditions including 5% CO_2, anaerobic, candle jar, and microaerophilic. We have also assessed media adjusted to the pH values (before filtration) 7.15, 7.25, 7.35, 7.4, 7.55, and 7.75.

Microscopy and examination of cultures

We used standard light microscopy to examine cultures. An Olympus IM or Olympus IMT-2 inverted microsope (the former fitted with a heating chamber) were set-up with a blue filter and raised condenser to provide optimal viewing conditions. Parasitic stages were easily identified using this system and cultures were examined daily at magnifications ranging from ×150 to ×600.

Quantitation of growth

A semiquantitative method was developed to assess growth of *C. parvum*. Cell monolayers grown in 24-well plates were inoculated with 5,000 oocysts/well and after incubation in RPMI-1640bm for 80–90 min, replaced with different medium formulations and incubated for 72 hr. Growth was quantified in duplicate wells after wells had been

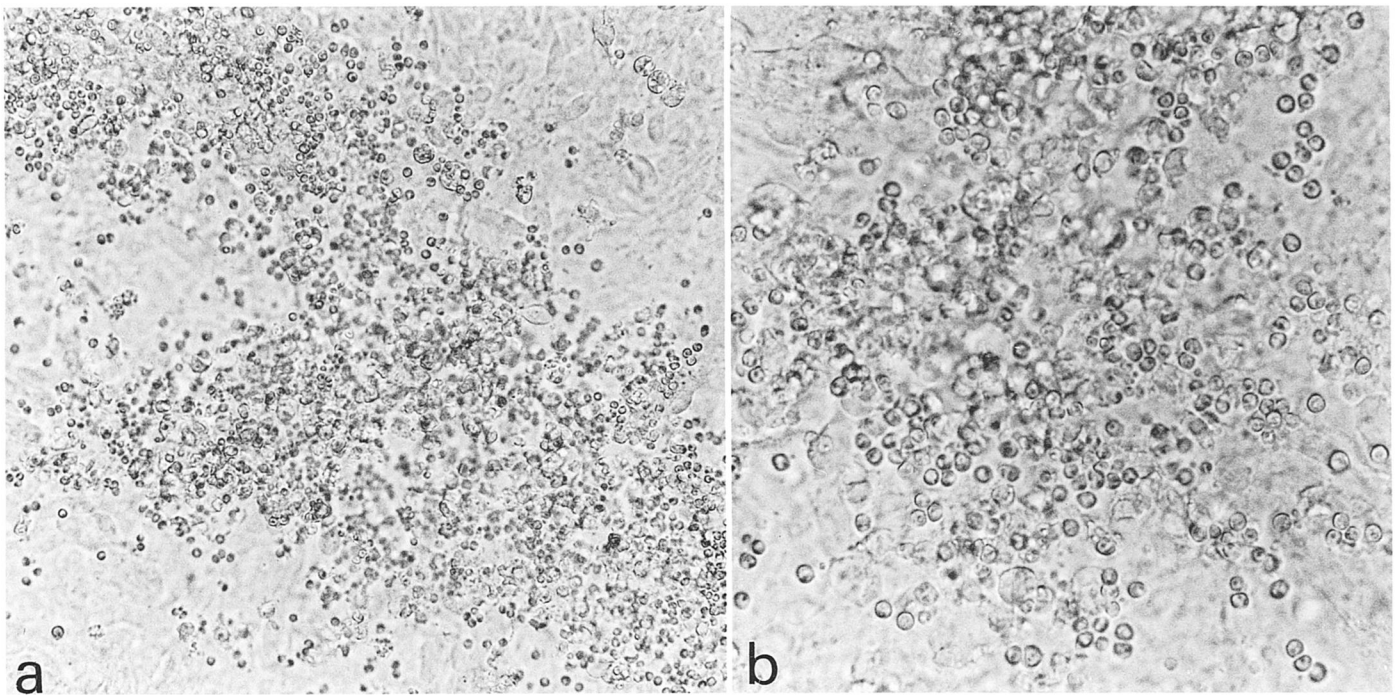

FIGURE 1. Light photomicrographs of developmental stages of *C. parvum* in HCT-8 cells in vitro 48 hr after infection. Medium used was optimized RPMI-1640 (see text) and incubation was in a candle jar. Magnification: (a) ×200; (b) ×400.

washed 2 times in PBS, filled with 1 ml Hank's balanced salt solutiuon, and scoring the absence or presence of 1 or more endogenous stages in 5 random fields, 6 times per well at ×600 magnification, i.e., 30 fields per well, 60 fields per growth condition tested. Growth data were analyzed using the nonparametric Mann–Whitney *U*-test.

RESULTS

Mouse model and oocyst purification

Using our mouse model and a Ficoll gradient purification method for obtaining oocysts we routinely obtained between 3–6 million and up to 15 million oocysts/mouse. Oocyst recovery rates from mouse fecal/intestinal homogenates were found to be between 65 and 85%. Phase-contrast examination of purified oocysts revealed clean preparations with minimal or no contamination with debris, bacteria, or yeast.

We have infected neonatal mice (6–8 days) with oocysts isolated from 15 different human fecal samples (Table I). We found that oocysts from 1 of these isolates (H2) produced a high oocyst excretion rate comparable to that obtained from the calf isolate (C1) used in this study, whereas the remaining samples produced no or low oocyst excretion rates in mice (Table I).

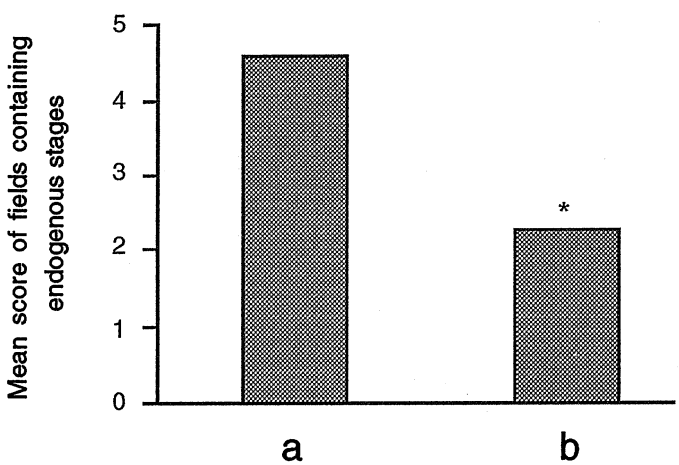

FIGURE 2. Comparison of growth of *C. parvum* after 72 hr: (a) optimized RPMI-1640 (see text) incubated in a candle jar; and (b) as described by Upton et al. (1995) using 5% CO_2 (*$P < 0.001$). Growth was assessed by scoring the absence or presence of 1 or more endogenous stages in 5 fields, 6 times in each of 2 wells using a light microscope at ×600 magnification.

In vitro cultivation

Using RPMI-1640bm, we tested 6 cell lines (MDCK, Caco-2, HF, HT-29, LIM1215, and HCT-8) and found that the human cecal adenocarcinoma cell line HCT-8 produced the highest parasite numbers in vitro. We observed that confluent HCT-8 monolayers more than 2 days old, and monolayers with less than 100% confluency did not produce comparable numbers of endogenous stages to those obtained with recently confluent monolayers.

Many of the media supplements described above were used alone, in combination, or both, and although not all combinations at different atmospheres and pH were tested, we have formulated a medium and incubation conditions that provide optimal growth. The optimum medium composition and incubation conditions were as follows: RPMI-1640 supplemented with sodium bicarbonate 0.3 g, bovine bile 0.02 g, glucose 0.1 g, folic acid 25 μg, 4-aminobenzoic acid 100 μg, calcium pantothenate 50 μg, ascorbic acid 875 μg, per 100 ml, pH 7.4 (before filtration), 1% FBS, and incubate in candle jar at 37 C, with

medium changes every 48 hr (Fig. 1). This formulation replaced RPMI-1640bm for resuspending oocysts after pretreatment in trypsin and was used to infect cell monolayers directly.

We have compared growth of *C. parvum* in our optimized medium and in that described by Upton et al. (1995) grown in a candle jar and in a 5% CO_2 incubator. We observed superior growth of *C. parvum* in our optimized medium with incubation in a candle jar compared to growth in medium and conditions described by Upton et al. (1995) (Fig. 2) and found that peak numbers of endogenous stages occurred between 48 and 72 hr. Although growth in our optimized medium was not significantly different in cultures incubated in a candle jar or in 5% CO_2, we consistently observed better growth in cultures incubated in candle jars. Incubating cultures in anaerobic or microaerophilic atmospheres resulted in poor growth of *C. parvum.*

DISCUSSION

In this study, best oocyst yields were obtained from mice aged between 7 and 8 days at the time of infection and by collecting fecal samples before harvesting oocysts from the gastrointestinal tract on day 8 PI. Current (1990) also recommended harvesting oocysts from the gut of infected mice on day 8 PI, and a study by Sinski et al. (1992) using mice infected at 3–6 days of age produced peak intestinal oocyst numbers 8 days PI.

Experimentally infected neonatal mice have been used to study the life cycle, development, and infectivity of *C. parvum* (Sherwood et al., 1982; Current and Reese, 1986; Ernest et al., 1986; Scaglia et al., 1991; Novak and Sterling, 1991; Sinski et al., 1992), to assess oocyst viability (Sherwood et al., 1982; Fayer et al., 1991), to test drugs against the parasite (Tzipori et al., 1982; Angus et al., 1984; Blagburn et al., 1991; Fayer and Ellis, 1993; Rohlman et al., 1993), and in immunological studies of cryptosporidiosis (Moon et al., 1988; Harp and Whitmire, 1991). Although Current (1990) described a technique for isolating oocysts from the gut of infected mice, this is the first study that describes a technique in which oocysts have been obtained from mice, further purified using a simple procedure, and subsequently used for the reproducible establishment of in vitro cultures.

Although the number of oocysts generated using experimentally infected mice in the present study (3–15 million oocysts/mouse; after purification) is less than the number usually shed by infected neonatal calves (1–10 million oocysts/g of feces for 1 wk; Angus, 1990), it provides ample material for in vitro cultivation and small-scale molecular (Morgan et al., 1995) and biochemical studies. In addition, the smaller oocyst yields obtainable using mice are compensated for by the ease in handling, maintaining, and processing material from mice infected with *C. parvum* compared to calves and other large animals.

The oocyst purification method is simple to perform, inexpensive, and was found to be very reliable. The Ficoll gradients can be prepared in advance and stored at 4 C for up to 6 mo without any loss in performance. Oocyst preparations are very clean and when used for in vitro cultivation do not result in bacterial or yeast contamination. Purified oocysts stored in PBS were successfully used to infect mice and susceptible cell monolayers after 6 mo storage. We have noticed, however, that oocyst storage results in a gradual decline in the efficiency of oocysts to produce endogenous stages in vitro compared to fresh oocysts and, therefore, rountinely use oocysts less than 6 mo old for cultivation. Storage in PBS also enables oocysts to be used for in vivo and in vitro infections, as well as DNA and biochemical procedures directly without the necessary wash procedures required when working with oocysts stored in dichromate.

The variation in infectivity of isolates of *C. parvum* in mice is likely to be a reflection of the differences in host specificity of the parasite. This is supported by the results of genetic characterization, which revealed that the human *C. parvum* isolate H2, which produced high oocyst excretion rates in mice was more closely related to calf isolates (including C1) than to other closely related human isolates of *C. parvum* (H1, H4, H5, H 6, H10, H11; Morgan et al., 1995) that produced low, or no, infections in mice. Other cross-transmission studies have also revealed differences and similarities in infectivity and virulence of calf and human isolates of *C. parvum* in different hosts (Current et al., 1983; Current and Reese, 1986; Pozio et al., 1992). However, this is the first study to compare the infectivity of *C. parvum* in mice using genetically characterized isolates and to show that genetically related isolates behave similarly in vivo. These findings also support our initial suggestion (Morgan et al., 1995) that different isolates of *C. parvum* are likely to vary in their zoonotic potential.

We used oocysts that had been exposed to a simple pretreatment step and resuspended in media containing bile to infect cell monolayers. This procedure has 3 advantages over using sporozoites to infect cells: (1) it is simple to perform and requires only 1 centrifugation step; (2) sporozoites have not been exposed to undue stress such as filtration and centrifugation associated with sporozoite purification procedures; and (3) once sporozoites are released from the oocyst they are in immediate contact with host cells. Upton, Tilley, and Brillhart (1994a, 1994b, 1995) and Upton, Tilley, Nesterenko, and Brillhart (1994) used a Clorox pretreatment of oocysts before infecting cells for the same reason as discussed above; however, their procedure requires additional centrifugation steps to remove the Clorox from the oocysts before infecting cells.

We found that FBS concentrations above 2% were inhibitory and resulted in a reduced growth of *C. parvum* with increasing concentrations, in addition to causing host cell overgrowth and making visualization of the endogenous stages difficult. Interestingly, omission of serum from the medium usually resulted in an increase in parasite numbers compared to cultures with serum, even after 72 hr, which is in contrast to the findings of Gut et al. (1991). However, the omission of serum from cultures also resulted in a slight deterioration of the host cell monolayer and, therefore, to reduce this effect, 1% FBS was added to the optimum medium formulation.

The removal of oocysts 80–90 min after infecting cells was not necessary and does not inhibit development of *C. parvum,* which is in contrast to the findings of Eggleston et al. (1994). Under our conditions, the pH of the medium usually remains alkaline even after 72 hr, unlike the medium described by Upton et al. (1995), which changed to an acidic pH after 24 hr, due likely to the high serum (10%) and glucose (11 g/L) concentration.

In our growth studies, we used between 2,000 and 5,000 oocysts/cm^2 of culture area, which is far fewer oocysts (or sporozoites) than described in other studies (on the order of 10,000–1,000,000 oocyst/cm^2; Current and Haynes, 1984; Flanigan et

al., 1991; Rasmussen et al., 1993; Eggleston et al., 1994; Upton, Tilley, and Brillhart, 1994a, 1994b, 1995; Upton, Tilley, Nesterenko, and Brillhart, 1994; Woods et al., 1995), and we still achieved high cell infection rates. We consistently obtained infection rates of between 30 and 60% after 2–4 days growth. Higher infection rates were achieved with higher oocyst doses. Growth of a human isolate (H2) of *C. parvum* produced a similar level of infection and development as the calf isolate, C1. It was observed, however, that optimal conditions for growth of *C. parvum* in HCT-8 cells were not the ideal conditions for growing the parasite in other cell lines. Therefore, it is essential that optimal culture conditions be determined for growing *C. parvum* in different cell types.

Although no attempt was made to quantify the different endogenous stages present during in vitro growth, we identified type 1 meronts, macrogamonts, microgamonts, and immature stages, and have also observed merozoites being released from meronts and merozoites infecting cells. We did not identify oocysts, a result consistent with the finding of Upton et al. (1995) who reported limited oocyst production in HCT-8 cells. We would like to point out 3 observations of interest. One is that although numerous macrogamonts were produced during cultivation, we never observed 4 merozoites being released from a meront. This is surprising because it is known that the 4 merozoites contained in type 2 meronts give rise to macrogamonts (and microgamonts) (Fayer et al., 1990). Secondly, once merozoites were released from the meront they infected cells very quickly, usually within 30 sec, but often after only a few seconds. The penetration of the merozoite into the host cell was very rapid and did not appear to involve any prior attachment or adhesion to the cell. In addition, if a merozoite failed to infect a cell within 1 min, it invariably did not infect a cell thereafter. Finally, we consistently observed merozoites being released from meronts shortly after medium changes (1–15 min), and we believe this to be a result of the sudden rise in pH that occurs after adding fresh medium.

In this study, we described a mouse model that is easy to handle and maintain and, combined with a purification procedure, provides a relatively simple method for the establishment of the parasite in most laboratories. In addition, we have simplified and optimized in vitro cultivation methods. This was achieved by performing a 1-step excystation procedure for treating oocysts prior to inoculating cells, using supplemented RPMI-1640, reducing the serum concentration, and incubating cultures in a candle jar. Until an in vitro cultivation method is developed that allows for the production of large numbers of oocysts and continuous culture of *C. parvum*, the procedures outlined in this study will be of great benefit to current and future reseachers working on this intriguing parasite.

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